Original Article

# Characterization of neuraminidase inhibitor-resistant influenza A(H1N1)pdm09 viruses isolated in four seasons during pandemic and post-pandemic periods in Japan

**Emi Takashita, Seiichiro Fujisaki, Noriko Kishida, Hong Xu, Masaki Imai, Masato Tashiro, Takato Odagiri, the Influenza Virus Surveillance Group of Japan***

Laboratory of Influenza Virus Surveillance, Influenza Virus Research Center, National Institute of Infectious Diseases, Tokyo, Japan.
*Correspondence*: Takato Odagiri, Laboratory of Influenza Virus Surveillance, Influenza Virus Research Center, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashimurayama, Tokyo 208-0011, Japan.
E-mail: todagiri@nih.go.jp
*The full member list is in Appendix.

**Background/Objectives** Japan has the highest frequency of neuraminidase (NA) inhibitor use against influenza in the world. Therefore, Japan could be at high risk of the emergence and spread of NA inhibitor-resistant viruses. The aim of this study was to monitor the emergence of NA inhibitor-resistant viruses and the possibility of human-to-human transmission during four influenza seasons in Japan.

**Methods** To monitor antiviral-resistant A(H1N1)pdm09 viruses, we examined viruses isolated in four seasons from the 2008–2009 season through the 2011–2012 season in Japan by allelic discrimination, NA gene sequencing, and NA inhibitor susceptibility.

**Results** We found that 157 (1·3%) of 12 026 A(H1N1)pdm09 isolates possessed an H275Y substitution in the NA protein that confers about 400- and 140-fold decreased susceptibility to oseltamivir and peramivir, respectively, compared with 275H wild-type viruses. The detection rate of resistant viruses increased from 1·0% during the pandemic period to 2·0% during the post-pandemic period. The highest detection rate of the resistant viruses was found in patients who were 0–9 years old. Furthermore, among the cases with resistant viruses, the percentage of no known exposure to antiviral drugs increased from 16% during the pandemic period to 44% during the post-pandemic period, implying that suspected human-to-human transmission of the resistant viruses gradually increased in the post-pandemic period.

**Conclusions** A(H1N1)pdm09 viruses resistant to oseltamivir and peramivir were sporadically detected in Japan, but they did not spread throughout the community. No viruses resistant to zanamivir and laninamivir were detected.

**Keywords** Influenza A(H1N1)pdm09 virus, neuraminidase inhibitor resistant, neuraminidase inhibitor susceptibility.

## Introduction

During the 2007–2008 influenza season, oseltamivir-resistant former seasonal A(H1N1) viruses emerged in Europe[1] and became the majority of A(H1N1) viruses within a year. These oseltamivir-resistant A(H1N1) viruses possessed a mutation causing a histidine-to-tyrosine substitution at amino acid position 275 (N1 numbering, H275Y) in the neuraminidase (NA) protein. The NA of these viruses had a slightly higher activity and affinity for the substrate than that from previously circulating sensitive viruses.[2] Furthermore, some potentially permissive substitutions in the NA and the hemagglutinin (HA) protein in addition to the H275Y substitution were reported,[3,4] so that oseltamivir-resistant A

(H1N1) viruses may acquire the capacity for efficient human-to-human transmission. The global spread of oseltamivir-resistant A(H1N1) viruses was obliged to change the chemotherapeutic strategy and limited the therapeutic options for influenza. Consequently, the worldwide monitoring of antiviral-resistant viruses is important for therapeutic and public health measures. In particular, in Japan, four NA inhibitors, oseltamivir, peramivir, zanamivir, and laninamivir, are approved for chemotherapy against influenza and are prescribed with the highest frequency in the world.[5] Therefore, Japan is at high risk of the emergence of NA inhibitor-resistant viruses. Because updated surveillance information on antiviral-resistant viruses from Japan is desired, year-round surveillance for influenza antiviral

resistance has been conducted through the National Institute of Infectious Diseases, in cooperation with 74 local public health institutes in Japan.

We have reported oseltamivir-resistant A(H1N1)pdm09 viruses through a preliminary surveillance of NA inhibitor-resistant viruses during 2009–2010.[6] In the present study, we screened more than 12 000 A(H1N1)pdm09 viruses isolated during four influenza seasons, from the 2008–2009 season through the 2011–2012 season, to monitor NA inhibitor-resistant viruses, and we detected 157 (1·3%) H275Y mutant viruses exhibiting cross-resistance to oseltamivir and peramivir. We describe here the virological characteristics of the oseltamivir- and peramivir-resistant A(H1N1)pdm09 viruses, the prescription history of patients infected with these resistant viruses and clusters of resistant virus infection.

## Methods

### Viruses

Clinical specimens and the corresponding patient records were collected in 500 sentinel hospitals consisting of pediatrics and internal medicine between May 2009 and August 2012 as part of the National Epidemiological Surveillance of Infectious Diseases in Japan. Most specimens were collected at the first medical examination. Influenza A(H1N1)pdm09 viruses were isolated from the specimens in 74 local public health institutes using Madin–Darby canine kidney (MDCK) or human colon carcinoma (Caco-2) cells. About three isolates per week were used for allelic discrimination assay and/or partial NA gene sequencing in the local public health institutes. Approximately 5–10% of total isolates were propagated in MDCK cells and used for NA inhibition assay and/or full-length NA and HA gene sequencing in the National Institute of Infectious Diseases.

### Allelic discrimination assay

To detect the H275Y substitution in NA, the allelic discrimination assay was performed as previously described.[7] Briefly, supernatants of A(H1N1)pdm09 virus-infected cells were directly added to the one-step duplex RT-PCR mixture without RNA purification, and the reaction was performed using the QuantiTect Virus kit (Qiagen, Dusseldorf, Germany). Fluorescent signals were collected during the annealing and extension steps, and amplification data and endpoint data were analyzed. Detection sensitivity of the allelic discrimination assay is 7·5 copies/reaction[7], and the assay detects a mixed population of H275Y mutant viruses and 275H wild-type viruses. We considered all mixed population as H275Y.

### Sequence analysis

Viral RNA was prepared using the QIAamp Viral RNA kit (Qiagen). To screen for the H275Y substitution in NA, the partial NA gene between 676 and 1,111 (from the start codon) was amplified from the viral RNA as previously described.[7] The full-length NA gene was amplified from viral RNA using a plus-sense primer N1-F1 and a minus-sense primer N1-R1 (Table 1). The HA1 domain of the HA gene was amplified using a plus-sense primer H1HA1-BEGIN and a minus-sense primer H1-1106-1087R (Table 1). Nucleotide sequences were determined using the BigDye Terminator v3·1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) with Applied Biosystems 3730 DNA Analyzer. The sequencing primers are shown in Table 1. DNA sequence assembly and analysis were performed using Sequencher 4·9 software (Gene Codes Corporation, Ann Arbor, MI, USA). Amino acids are described with the N1 numbering.

### NA inhibition assay

An NA inhibition assay was performed to determine the susceptibility of viruses to NA inhibitors. Four NA inhibitors, oseltamivir carboxylate (F. Hoffmann-La Roche Ltd., Basel, Switzerland), zanamivir (GlaxoSmithKline, Stevenage, UK), peramivir (Shionogi & Co., Ltd., Osaka, Japan), and laninamivir (Daiichi Sankyo Co., Ltd., Tokyo, Japan) were kindly provided by the manufacturers. The susceptibility of viruses to NA inhibitors was determined by chemiluminescent

**Table 1.** Primers used for NA and HA gene sequencing of A(H1N1)pdm09 viruses

| Primer* | Sequence (5′–3′) |
| --- | --- |
| Primers for NA | |
| N1-F1 | AGCAAAAGCAGGAGTTCAAAATGA |
| N1-17-35F | AGATAATAACCATTGGTTC |
| N1-290-307F | GGGCTATATACAGTAAAG |
| N1-434-416R | GAATGTTTGTCATTTAGCA |
| N1-548-532R | GCACTTGCTGACCAAGC |
| N1-676-694F | ACACAAGAGTCTGAATGTG |
| N1-941-959F | TAGGATACATATGCAGTGG |
| N1-959-941R | CCACTGCATATGTATCCTA |
| N1-1111-1130F | TTTGAGATGATTTGGGATCC |
| N1-1130-1111R | GGATCCCAAATCATCTCAAA |
| N1-1394-1375R | AATGGCAACTCAGCACCGTC |
| N1-R1 | GTAGAAACAAGGAGTTTTTTGAAC |
| Primers for HA1 | |
| H1HA1-BEGIN | AGCAAAAGCAGGGGAAAATAA |
| H1-56-76F | CATTATGTATAGGTTATCATG |
| H1-277-296F | TGGTCCTACATTGTGGAAAC |
| H1-385-366R | CAAATGATGACACTGAGCTC |
| H1-596-578R | GGATGGTGAATGCCCCATA |
| H1-768-788F | AATAACATTCGAAGCAACTGG |
| H1-1106-1087R | TGATAACCGTACCATCCATC |

*Based on cRNA sequences of A/Narita/1/2009 virus.
Accession numbers for NA and HA genes are EPI179482 and EPI179438, respectively.

enzyme inhibition assay with the NA-Star Influenza Neuraminidase Inhibitor Resistance Detection kit (Applied Biosystems). Results were expressed as the drug concentrations required to inhibit NA activity by 50% ($IC_{50}$). The $IC_{50}$ values were calculated using MikroWin 2000 software (Mikrotek Laborsysteme GmbH, Overath, Germany) as previously described.[6] The WHO expert working group on surveillance of influenza antiviral susceptibility has defined criteria of NA inhibitor susceptibility based on the fold change of $IC_{50}$ values compared with reference $IC_{50}$ values.[8] For influenza A viruses, use of normal (<10-fold increase), reduced (10- to 100-fold increase), and highly reduced (>100-fold increase) inhibition, and for influenza B viruses, the same criteria but using <5-fold, 5- to 50-fold and >50-fold increases, is recommended.

### Statistical analysis

Box-and-whisker plots were used to identify extreme $IC_{50}$ values as previously described.[9] The box contains 50% of the results, representing the middle two quartiles (25–75%). The length of the box represents the interquartile range (IQR). The whiskers extend to the largest and smallest values in the central part of the distribution before the region containing outliers is reached. The mild (between 1·5 and 3·0 IQR from the 25th and 75th percentiles) and the extreme (>3·0 IQR from the 25th and 75th percentiles) outliers are defined. Differences between groups were evaluated by chi-square test. *P* values less than 0·05 were considered statistically significant.

### Phylogenetic analysis

Nucleotide sequences of the full-length NA gene and the HA1 domain of the HA gene were subjected to phylogenetic analysis. Multiple alignments and phylogenetic trees were constructed using the MEGA 5·0 software.[10] The phylogenetic trees were obtained by the neighbor-joining method with bootstrap analysis of 1000 replicates. Accession numbers for the sequences used in the trees are shown in Table 2.

## Results

### Detection of A(H1N1)pdm09 viruses with the H275Y substitution

During the 2008–2009 through the 2011–2012 influenza seasons, 38 292 cases of A(H1N1)pdm09 virus isolation/detection were reported in Japan (Figure 1A). We screened 12,026 isolates by allelic discrimination and/or partial NA gene sequencing to detect the H275Y substitution (Figure 1B). During the pandemic period, 10 (0·5%) of 2,168 isolates in the 2008-2009 season and 69 (1·1%) of 6,005 isolates in the 2009–2010 season possessed the H275Y substitution (Figure 1B,C). During the post-pandemic period, 78 (2·0%) of 3844 isolates in the 2010–2011 season were

H275Y mutants. In the 2011–2012 season, there were no outbreaks of the A(H1N1)pdm09 virus in Japan, and no H275Y mutant virus was detected from the sporadic cases of A(H1N1)pdm09 virus infection. The detection rates of H275Y mutant viruses increased from 1·0% during the pandemic period to 2·0% during the post-pandemic period. Most H275Y mutant viruses were detected in sporadic cases, and there were no geographical clusters of mutant virus in Japan.

### Susceptibility of A(H1N1)pdm09 viruses with the H275Y substitution to NA inhibitors

We analyzed 155 isolates in the 2008–2009 season, 685 isolates in the 2009–2010 season, 249 isolates in the 2010–2011 season, and eight isolates in the 2011–2012 season by the NA inhibition assay. The $IC_{50}$ values of A(H1N1)pdm09 viruses with the H275Y substitution to NA inhibitors are shown in Figure 2. In the 2008–2009 season, susceptibility of viruses to oseltamivir and zanamivir was analyzed (Figure 2A). Peramivir was approved in Japan in January 2010 and was included in the NA inhibition assay for isolates collected from the 2009-2010 season and later seasons (Figure 2B). Laninamivir was licensed in Japan in October 2010 and was included in the NA inhibition assay for isolates collected from the 2010-2011 season and the 2011–2012 season (Figure 2C). H275Y mutant viruses from throughout the pandemic and post-pandemic periods showed about 400- and 140-fold increased $IC_{50}$ values to oseltamivir and peramivir, respectively, compared with 275H. However, the $IC_{50}$ values of H275Y mutants to zanamivir and laninamivir were comparable to those of viruses with 275H. These results indicate that the H275Y mutant viruses exhibit resistance to both oseltamivir and peramivir, but are sensitive to zanamivir and laninamivir. Moreover, there was no significant difference in the $IC_{50}$ values to oseltamivir and peramivir among H275Y mutant viruses isolated in pandemic and post-pandemic periods.

### Age distribution of patients with oseltamivir- and peramivir-resistant A(H1N1)pdm09 viruses

The age distributions of patients infected with A(H1N1)pdm09 viruses are shown in Figure 3. The total number of infected patients peaked at 10–19 years of age in the 2008–2009 season (Figure 3A). During the 2009–2010 and 2010–2011 seasons, the total number of infected patients peaked at 0–9 years of age. The percentages of patients aged 0-9 years in the total number of patients with oseltamivir- and peramivir-resistant viruses were 50% in the 2008–2009 season, 65% in the 2009–2010 season, and 49% in the 2010–2011 season (Figure 3B). These results indicate that the majority of resistant viruses are detected in infants and young children. Moreover, the percentage of individuals aged 10–19 years and 50–59 to 60–69 years with resistant viruses

**Table 2.** Accession numbers for the sequences used in the phylogenetic trees

**Viral isolates_HA sequence_NA sequence**

A/AICHI/1019/2009_EPI232919_-
A/AICHI/1121/2009_EPI233018_-
A/AOMORI/18/2010_EPI272597_EPI272596
A/AOMORI/3/2010_EPI272391_EPI272390
A/CHIBA-C/41/2010_EPI277581_EPI277580
A/CHIBA/1067/2009_EPI278741_EPI278742
A/CHIBA/12/2010_EPI295380_EPI295379
A/EHIME/65/2009_EPI272576_EPI272575
A/FUKUI/67/2011_EPI321458_EPI321457
A/FUKUI/72/2011_EPI329091_EPI334477
A/FUKUOKA-C/34/2010_EPI272600_EPI272599
A/FUKUOKA/1/2010_EPI248527_EPI248526
A/GIFU/21/2011_EPI321474_EPI321473
A/HAMAMATU-C/7/2011_EPI321452_-
A/HIROSHIMA-C/26/2010_EPI272568_EPI272567
A/HIROSHIMA/17/2012_EPI378229_EPI378228
A/HIROSHIMA/18/2012_EPI378231_EPI378230
A/HIROSHIMA/49/2011_EPI316263_EPI318152
A/HIROSHIMA/66/2011_EPI321439_EPI329139
A/HOKKAIDO/12/2011_EPI316280_EPI318171
A/HOKKAIDO/93/2011_EPI321482_EPI321481
A/HYOGO/3017/2010_EPI278711_EPI278710
A/HYOGO/4012/2011_EPI320241_EPI320240
A/IBARAKI/103/2011_EPI321464_EPI321463
A/IBARAKI/115/2010_EPI277575_EPI277574
A/ISHIKAWA/566/2009_EPI240597_-
A/ISHIKAWA/70/2011_EPI321456_EPI321455
A/IWATE/1030/2010_EPI272571_EPI272570
A/KANAGAWA/75/2011_EPI329105_EPI329104
A/KAWASAKI/122/2011_EPI336870_EPI336869
A/KOBE/181/2010_EPI278693_EPI278692
A/KUMAMOTO-C/39/2011_EPI378237_EPI378236
A/KUMAMOTO/2/2010_EPI272399_EPI272398
A/KUMAMOTO/20/2010_EPI272582_EPI272581
A/MIYAGI/32/2011_EPI329100_EPI338384
A/MIYAZAKI/40/2011_EPI321462_EPI321461
A/NAGANO-C/152/2009_EPI240602_-
A/NAGANO-C/21/2012_EPI378227_EPI378226
A/NAGANO-C/82/2009_EPI230097_EPI230096
A/NAGANO/2289/2011_EPI329071_EPI334475
A/NAGOYA/1013/2010_EPI276934_EPI276933
A/NARA/39/2010_EPI291745_EPI291744
A/NARA/59/2011_EPI321490_EPI321489
A/NARITA/1/2009_EPI179438_EPI179482
A/NIIGATA-C/186/2009_EPI272415_EPI272414
A/NIIGATA/1813/2009_EPI272411_EPI272410
A/NIIGATA/529/2010_EPI272609_EPI272608
A/OITA/126/2009_EPI226296_EPI226295
A/OITA/49/2010_EPI272588_EPI272587
A/OITA/57/2010_EPI272612_EPI272611
A/OKINAWA/40/2011_EPI320274_EPI320273
A/OSAKA-C/20/2010_EPI272585_EPI272584
A/OSAKA-C/2004/2011_EPI329081_EPI329080
A/OSAKA/52/2011_EPI320269_EPI320268
A/OSAKA/58/2010_EPI272618_EPI272617

**Table 2.** (*Continued*)

**Viral isolates_HA sequence_NA sequence**

A/OSAKA/60/2010_EPI279612_EPI286663
A/SAITAMA/160/2011_EPI329078_EPI329077
A/SAITAMA/289/2011_EPI329107_EPI334515
A/SAITAMA/319/2011_EPI348493_EPI348492
A/SAITAMA/80/2012_EPI378239_EPI378238
A/SAPPORO/190/2009_EPI230125_EPI230124
A/SAPPORO/163/2011_EPI329106_EPI334513
A/SAPPORO/31/2010_EPI254533_EPI254532
A/SAPPORO/4/2012_EPI356998_EPI356997
A/Sendai-H/41/2010_EPI240607_-
A/SENDAI/151/2009_EPI287187_EPI287186
A/SHIMANE/1062/2009_EPI234600_-
A/SHIMANE/164/2011_EPI329051_EPI329050
A/SHIZUOKA-C/38/2010_EPI272421_-
A/SHIZUOKA/59/2012_EPI378233_EPI378232
A/TOCHIGI/34/2011_EPI329049_EPI329048
A/TOCHIGI/87/2010_EPI272627_EPI272626
A/TOKUSHIMA/2/2009_EPI214013_EPI214014
A/TOKYO/11203/2011_EPI329098_EPI329097
A/TOKYO/91256/2009_EPI248546_EPI248545
A/TOTTORI/225/2009_EPI248548_EPI248547
A/WAKAYAMA-C/19/2010_EPI276936_EPI276935
A/WAKAYAMA/23/2011_EPI316261_EPI318149
A/WAKAYAMA/34/2010_EPI272591_EPI272590
A/WAKAYAMA/8/2011_EPI320251_EPI320250
A/YAMAGATA/206/2011_EPI333154_EPI333153
A/YAMAGATA/752/2009_EPI241773_-
A/YAMAGUCHI/23/2010_EPI272639_EPI272638
A/YOKOHAMA/1538/2009_EPI244285_EPI244284

increased in the post-pandemic period rather than in the pandemic period.

## Prescription history of patients with oseltamivir- and peramivir-resistant A(H1N1)pdm09 viruses

The prescription history of patients with H275Y mutant viruses is shown in Table 3. The majority of H275Y mutant viruses was detected from the patients during treatment or prevention with oseltamivir (80% and 81% during the pandemic periods, 2008–2009 and 2009–2010 seasons, respectively, and 42% during the post-pandemic period, 2010–2011 season). Eight percent of the total H275Y mutants were detected from the patients that had been treated with peramivir in the 2010–2011 season. Noteworthily, 40% in the 2008–2009 season, 12% in the 2009–2010 season, and 18% in the 2010–2011 season of the total H275Y mutants were detected from the prophylactic use of oseltamivir with a half dose (75 mg once daily).

Among the cases of H275Y mutant virus infections, the percentage of no known exposure to antiviral drugs significantly increased from 16% during the pandemic period to 44% during the post-pandemic period ($P < 0.05$), suggesting
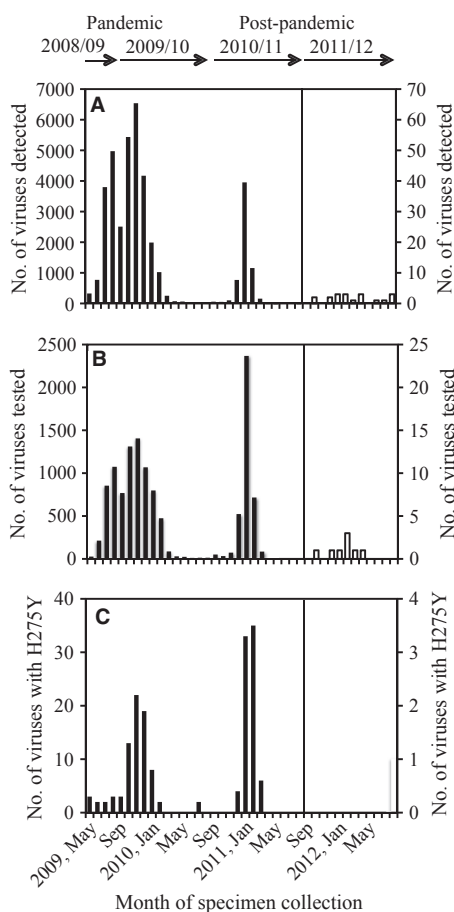
**Figure 1.** Detection of A(H1N1)pdm09 viruses with the H275Y substitution during the 2008–2012 influenza seasons in Japan. (A) Monthly reports of A(H1N1)pdm09 virus isolation/detection by local public health institutes under the National Epidemiological Surveillance of Infectious Diseases. (B) Number of A(H1N1)pdm09 viruses tested in this study. (C) Number of A(H1N1)pdm09 viruses with the H275Y substitution. Closed bars relate to the left *y*-axis and open bars to the right *y*-axis.



**Figure 2.** Susceptibility of A(H1N1)pdm09 viruses with the H275Y substitution to NA inhibitors. $IC_{50}$ values of A(H1N1)pdm09 viruses to oseltamivir, zanamivir, peramivir, and laninamivir were determined by NA inhibition assay. Viruses were from the 2008–2009 (A), the 2009–2010 (B), and the 2010–2011 (C) influenza seasons. Box-and-whisker plots of $IC_{50}$ values are shown. Numbers on top of each box-and-whisker plot indicate the mean $IC_{50}$ value $\pm$ 1 standard deviation.

that human-to-human transmission cases of the resistant viruses had gradually increased. In fact, two clusters of resistant virus infection with suspected human-to-human transmission were independently observed. The first cluster occurred in a facility for handicapped individuals in eastern Japan in January–February 2011. In this cluster, resistant viruses with the H275Y mutation were detected in three patients who received prophylaxis with oseltamivir; 1 week later, the resistant viruses were detected in three patients who were not exposed to antivirals. The HA, NA, and matrix (M) genes of the six resistant viruses were sequenced and confirmed to possess the same amino acid sequences, implying that a single H275Y mutant virus circulated among the patients in the facility. The second cluster of cases occurred in a hospital in western Japan in February–March 2011. In this cluster, resistant viruses with the H275Y mutation were detected in three immunocompromised
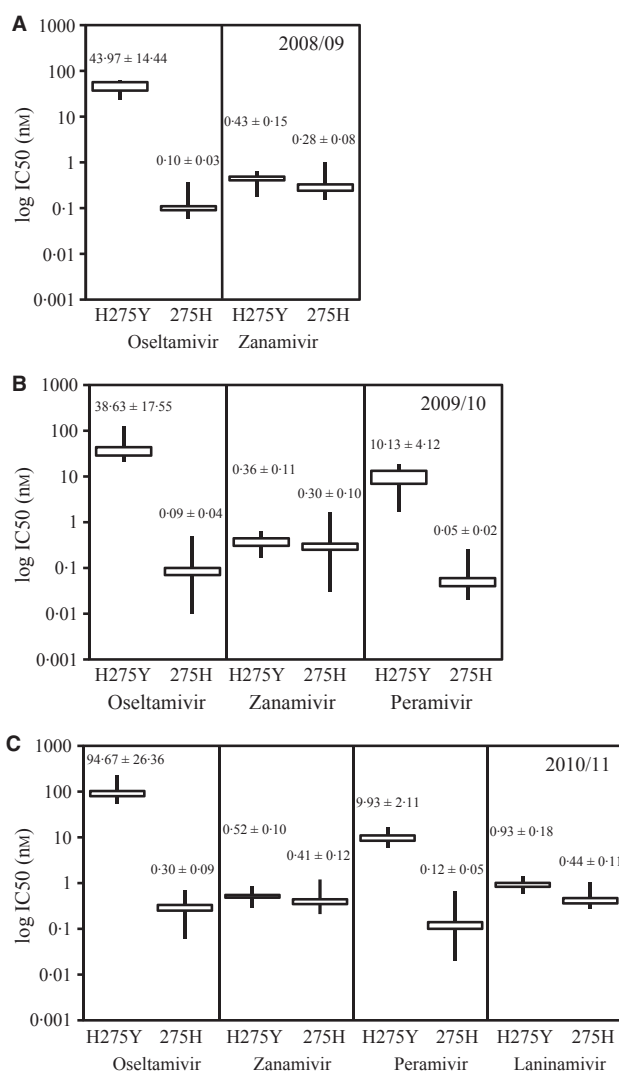
inpatients who had received prophylaxis with oseltamivir and was then later detected in a nurse. These four viruses possessed the same HA and NA amino acid sequences. However, there is no evidence of widespread outbreaks of the resistant viruses in the community, although the number of suspected cases of human-to-human transmission of drug-resistant viruses increased.

## Phylogenetic analyses of the NA and HA1 genes of A (H1N1)pdm09 viruses

Representative isolates from the 2008–2009 season through the 2011–2012 season were used for the phylogenetic
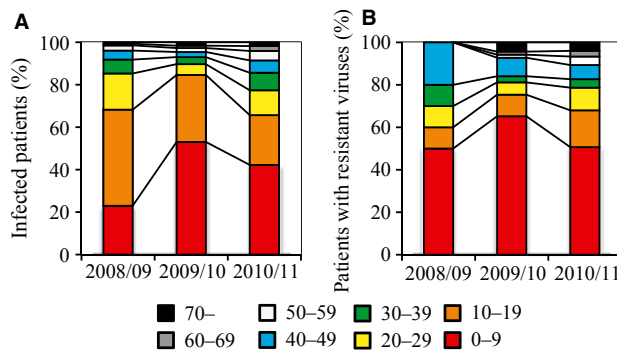
**Figure 3.** Age distribution of patients with oseltamivir- and peramivir-resistant A(H1N1)pdm09 viruses. Age distribution of patients during the 2008–2009, the 2009–2010, and the 2010–2011 influenza seasons. (A) The proportion of patients infected with A(H1N1)pdm09 viruses. (B) The proportion of patients with resistant viruses.

analysis. Viruses isolated in the 2009–2010 (yellow), 2010–2011 (orange), and 2011–2012 (blue) seasons contained NA genes that belonged to the N248D clade (Figure 4A). The 2010–2011 and 2011–2012 isolates fell into the V241I and N369K subclades. Viruses isolated in the 2009–2010 (yellow), 2010–2011 (orange), and 2011–2012 (blue) seasons contained HA1 genes that fell into S203T clade (Figure 4B). The 2010–2011 and 2011–2012 isolates tended to fall into the S143G subclade. The H275Y mutant viruses did not form any certain clade in the NA and the HA1 phylogenetic tree.

## Discussion

The highest frequency of antiviral drug use throughout the world has been recorded in Japan, ever since the NA inhibitors zanamivir and oseltamivir were licensed and covered by national health insurance in Japan in 2000 and

2001, respectively.[5] Consequently, surveillance information on antiviral-resistant viruses from Japan is valued by clinical and public health sectors throughout the world. We screened 12 026 A(H1N1)pdm09 viruses isolated in Japan from the 2008–2009 season through the 2011–2012 season and detected 157 (1·3%) resistant viruses with the H275Y substitution in the NA protein. These H275Y mutant viruses were confirmed to be resistant to both oseltamivir and peramivir, but sensitive to zanamivir and laninamivir, which is consistent with previous findings that oseltamivir-resistant viruses exhibit cross-resistance to peramivir.[11,12] The detection rates of the resistant viruses were 0·5% in the 2008–2009 season, 1·1% in the 2009–2010 season, and 2·0% in the 2010-2011 season in Japan. These detection rates were similar to the rates detected by the surveillance of NA inhibitor-resistant former seasonal A(H1N1) viruses collected in Japan during the 1999–2002,[9] the 2003–2007,[5] and the 2007–2008 seasons.[13]

In our study of A(H1N1)pdm09 viruses, the proportion of patients with oseltamivir- and peramivir-resistant viruses among those who were 0–9 years of age was high. The administration of oseltamivir to patients aged 10–19 years has been restricted by the Ministry of Health, Labour and Welfare of Japan, since 2007 due to reports of abnormal behavior after oseltamivir treatment. The percentages of patients aged 0–9 and 10–19 years in the total number of patients prescribed with NA inhibitors are 39·3 and 18·1% in the 2008–2009 season, 36·9 and 33·8% in the 2009–2010 season, and 37·8 and 19·3% in the 2010–2011 season, respectively. Our finding is consistent with the previous cumulative data from clinical trials that the incidence of oseltamivir-resistant viruses was 0·32% in adults aged 18–65 years and 4·1% in children aged 1–12 years.[14] The higher incidence of resistant viruses among children appears to be

**Table 3.** Prescription history of patients with A(H1N1)pdm09 viruses with the H275Y substitution

| Prescription history | 2008–2009 season | | 2009–2010 season | | 2010–2011 season | |
| --- | --- | --- | --- | --- | --- | --- |
| | No. of viruses tested | No. of viruses with H275Y | No. of viruses tested | No. of viruses with H275Y | No. of viruses tested | No. of viruses with H275Y |
| Oseltamivir* | 56 (4) | 8 (4) | 570 (9) | 56 (8) | 72 (16) | 33 (14) |
| Zanamivir* | 27 | 0 | 91 (1) | 0 | 24 | 1 |
| Peramivir | 0 | 0 | 0 | 0 | 14 | 6 |
| Laninamivir | 0 | 0 | 0 | 0 | 5 | 0 |
| Oseltamivir and peramivir | 0 | 0 | 0 | 0 | 7 | 2 |
| Oseltamivir and zanamivir | 0 | 0 | 20 | 2 | 0 | 0 |
| Peramivir and zanamivir | 0 | 0 | 0 | 0 | 1 | 0 |
| No known exposure to drug | 888 | 2 | 1379 | 11 | 250 | 34 |
| Insufficient information | 1197 | 0 | 3945 | 0 | 3471 | 2 |
| Total | 2168 | 10 | 6005 | 69 | 3844 | 78 |

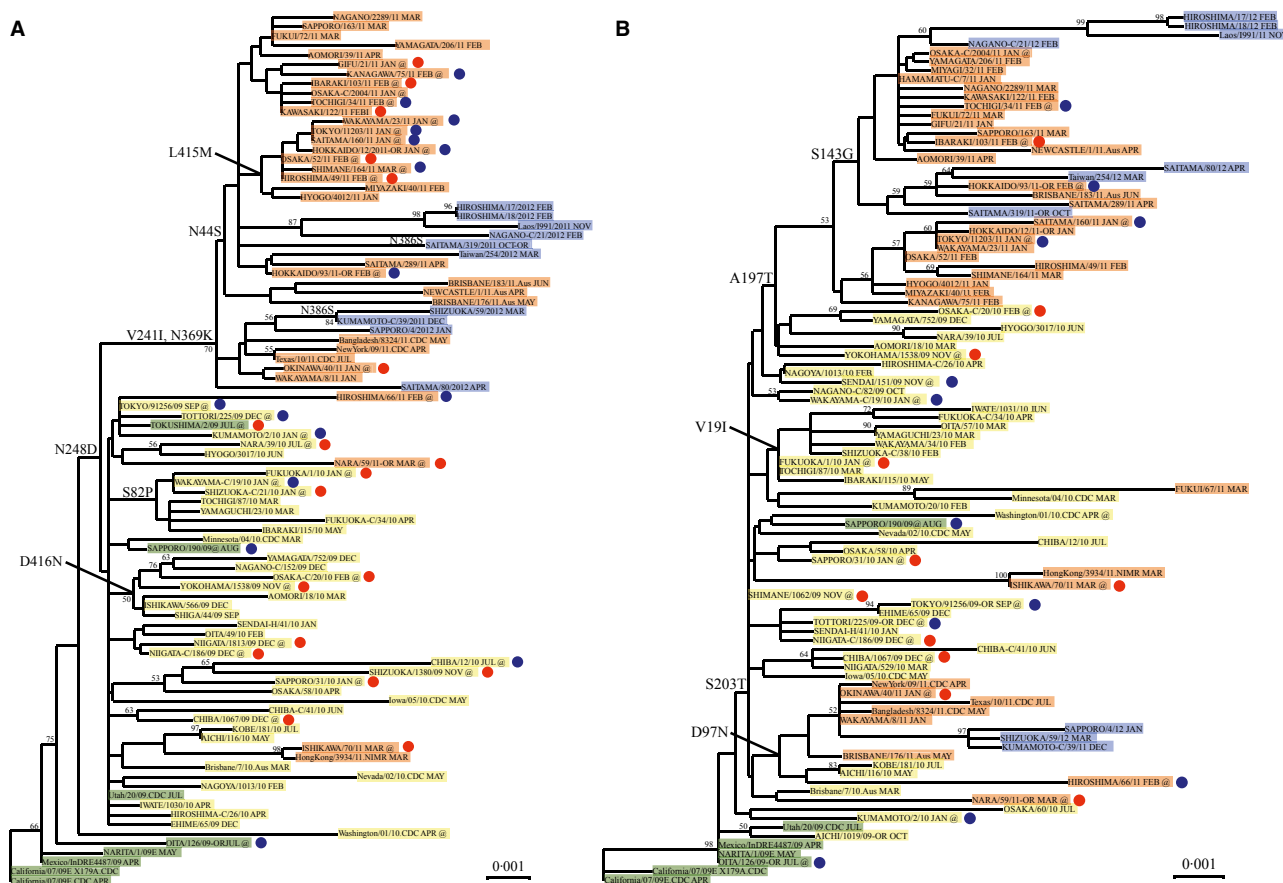*Cases who received prophylaxis are shown in parentheses.

**Figure 4.** Phylogenetic trees of the A(H1N1)pdm09 virus NA and HA1 genes. Phylogenetic trees of NA (A) and HA1 (B) genes were constructed by the neighbor-joining method with bootstrap values of 1000 replicates. Amino acid substitutions relative to the A/California/07/2009 virus are shown on the left side of the node. Bootstrap values are indicated only when greater than 50%. Viruses isolated in the 2008–2009, the 2009–2010, the 2010-2011, and the 2011–2012 influenza seasons are colored in green, yellow, orange, and blue, respectively. The blue circles indicate the H275Y mutant viruses in the cases with no known exposure to antiviral drug. The H275Y mutant viruses in the cases associated with antiviral drug use are represented by red circles. The scale bar shows the nucleotide substitutions per site.

related to longer periods of viral shedding and higher levels of viral titers.[15]

Based on clinical history information, resistant virus infections were predominantly associated with the therapeutic use of oseltamivir or peramivir. However, 12–40% of the total resistant viruses were detected from patients under prophylaxis of oseltamivir with a subtherapeutic dose (75 mg once daily), but not a therapeutic dose (75 mg twice daily) as observed in our previous study.[6] Most resistant viruses from the prophylactic use emerged sporadically, although a suspected human-to-human transmission case from a patient under treatment to a patient under prophylaxis was observed. The emergence of resistant viruses in patients who received prophylaxis cannot be disregarded, so that the prophylactic use of antivirals is generally not recommended and should be followed by monitoring for emergence of resistant virus. In Japan, the Japanese Association for Infectious Diseases has recommended post-exposure

prophylaxis in hospitals and nursing homes, following the recommendations of the Advisory Committee on Immunization Practices, USA[16], and the Health Protection Agency, UK.[17] Consequently, the emergence of antiviral-resistant viruses from the prophylactic use should be paid particular attention.

Among the cases with resistant viruses, the percentage of no known exposure to antiviral drugs significantly increased from 16% during the pandemic period to 44% during the post-pandemic period. A similar increase was observed in other continents.[18–22] These observations may suggest that limited human-to-human transmission with H275Y mutant viruses has gradually increased in the post-pandemic periods. We observed suspected human-to-human transmission in two independent in-facility infection clusters that occurred in eastern and western Japan in 2011. The former cluster occurred in a facility for handicapped individuals. H275Y mutant viruses with the same HA, NA, and M amino acid

sequences were initially detected in patients who received prophylaxis with oseltamivir; 1 week after these initial infections, infections were detected in individuals who did not receive treatment or prophylaxis with antiviral drugs. In this cluster of cases, it appears that a single H275Y mutant circulated inside the facility and was transmitted among the patients. The second cluster of cases occurred in a hospital. The H275Y mutant viruses were initially detected in immunocompromised inpatients who had received prophylaxis with oseltamivir and then in a nurse. These H275Y mutant viruses possessed the same HA and NA amino acid sequences, suggesting human-to-human transmission of the mutant virus. These two suspected clusters of human-to-human transmission did not spread beyond the residential facility or hospital to the outside community. However, the increasing number of H275Y mutant infections among those with no known exposure to antivirals is a warning that continued surveillance to monitor human-to-human transmission of NA inhibitor-resistant viruses is necessary worldwide; this is especially true as we had seen that oseltamivir-resistant former seasonal A(H1N1) viruses with the H275Y mutation, which emerged in Europe in the 2007–2008 season, spread globally within a year.[1]

Some potentially permissive substitutions together with the H275Y mutation in the NA protein were reported for A (H1N1) viruses[3,4] and A(H1N1)pdm09 viruses.[20,22] In the case of A(H1N1) viruses, R222Q and V234M (N2 numbering) substitutions in NA restored the surface-expressed NA activity,[3] and these substitutions occurred before the global emergence of H275Y mutant viruses. Three substitutions (T82K, K141E, and R189K) in the HA protein promoted virus replication in the presence of the H275Y substitution in NA.[4] In the case of A(H1N1)pdm09 viruses, three substitutions (V241I, N369K, and N386S) in the NA protein may offset the destabilizing effect of the H275Y substitution.[20] In our study, the H275Y mutant viruses did not possess these permissive substitutions and did not form any certain clade in the NA and the HA1 phylogenetic trees (Figure 4A,B). However, oseltamivir-resistant A(H1N1)pdm09 viruses detected in a cluster of community cases in Australia contained the potentially permissive mutations in NA.[20] Therefore, sustained monitoring of the NA and HA gene sequences together with antiviral susceptibility assays is important to explore the possibility of human-to-human transmission of NA inhibitor-resistant viruses.

## Acknowledgements

## Conflict of interests

The authors declare that they have no conflict of interests.

## References

1  Lackenby A, Hungnes O, Dudman SG et al. Emergence of resistance to oseltamivir among influenza A(H1N1) viruses in Europe. Euro Surveill 2008; 13:5.

2  Rameix-Welti MA, Enouf V, Cuvelier F, Jeannin P, van der Werf S. Enzymatic properties of the neuraminidase of seasonal H1N1 influenza viruses provide insights for the emergence of natural resistance to oseltamivir. PLoS Pathog 2008; 4:e1000103.

3  Bloom JD, Gong LI, Baltimore D. Permissive secondary mutations enable the evolution of influenza oseltamivir resistance. Science 2010; 328:1272–1275.

4  Ginting TE, Shinya K, Kyan Y et al. Amino acid changes in hemagglutinin contribute to the replication of oseltamivir-resistant H1N1 influenza viruses. J Virol 2012; 86:121–127.

5  Tashiro M, McKimm-Breschkin JL, Saito T et al. Surveillance for neuraminidase-inhibitor-resistant influenza viruses in Japan, 1996–2007. Antivir Ther 2009; 14:751–761.

6  Ujike M, Ejima M, Anraku A et al. Monitoring and characterization of oseltamivir-resistant pandemic (H1N1) 2009 virus, Japan, 2009–2010. Emerg Infect Dis 2011; 17:470–479.

7  Nakauchi M, Ujike M, Obuchi M et al. Rapid discrimination of oseltamivir-resistant 275Y and -susceptible 275H substitutions in the neuraminidase gene of pandemic influenza A/H1N1 2009 virus by duplex one-step RT-PCR assay. J Med Virol 2011; 83:1121–1127.

8  World Health Organization. Meetings of the WHO working group on surveillance of influenza antiviral susceptibility-Geneva, November 2011 and June 2012. Wkly Epidemiol Rec 2012; 87:369–374.

9  Monto AS, McKimm-Breschkin JL, Macken C et al. Detection of influenza viruses resistant to neuraminidase inhibitors in global surveillance during the first 3 years of their use. Antimicrob Agents Chemother 2006; 50:2395–2402.

10  Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011; 28:2731–2739.

11  Baum EZ, Wagaman PC, Ly L et al. A point mutation in influenza B neuraminidase confers resistance to peramivir and loss of slow binding. Antiviral Res 2003; 59:13–22.

12  Baz M, Abed Y, Boivin G. Characterization of drug-resistant recombinant influenza A/H1N1 viruses selected in vitro with peramivir and zanamivir. Antiviral Res 2007; 74:159–162.

13  Ujike M, Shimabukuro K, Mochizuki K et al. Oseltamivir-resistant influenza viruses A (H1N1) during 2007–2009 influenza seasons Japan. Emerg Infect Dis 2010; 16:926–935.

14  Aoki FY, Boivin G, Roberts N. Influenza virus susceptibility and resistance to oseltamivir. Antivir Ther 2007; 12:603–616.

15  Kiso M, Mitamura K, Sakai-Tagawa Y et al. Resistant influenza A viruses in children treated with oseltamivir: descriptive study. Lancet 2004; 364:759–765.

16  Fiore AE, Fry A, Shay D, Gubareva L, Bresee JS, Uyeki TM. Antiviral agents for the treatment and chemoprophylaxis of influenza;

recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep 2011; 60:1–24.

17 Health Protection Agency: Pharmacological treatment and prophylaxis of influenza. January 2011. Available at: http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1287147812045 (Accessed 30 May 2013).

18 Lackenby A, Moran GJ, Pebody R et al. Continued emergence and changing epidemiology of oseltamivir-resistant influenza A(H1N1) 2009 virus, United Kingdom, winter 2010/11. Euro Surveill 2011; 16:19784.

19 Wang B, Taylor J, Ratnamohan M et al. Frequency of oseltamivir resistance in Sydney, during the Newcastle outbreak of community transmitted oseltamivir-resistant influenza A(H1N1)pdm09 virus, Australia, June to August 2011. Euro Surveill 2012; 17:20210.

20 Hurt AC, Hardie K, Wilson NJ et al. Characteristics of a widespread community cluster of H275Y oseltamivir-resistant A(H1N1)pdm09 influenza in Australia. J Infect Dis 2012; 206:148–157.

21 Storms AD, Gubareva LV, Su S et al. Oseltamivir-resistant pandemic (H1N1) 2009 virus infections, United States, 2010-11. Emerg Infect Dis 2012; 18:308–311.

22 Meijer A, Jonges M, van Beek P et al. Oseltamivir-resistant influenza A (H1N1)pdm09 virus in Dutch travellers returning from Spain, August 2012. Euro Surveill 2012; 17:20266.

## Appendix

Members of the Influenza Virus Surveillance Group of Japan are as follows: Rika Komagome (Hokkaido Institute of Public Health), Masayuki Kikuchi (Sapporo City Institute of Public Health), Rika Tsutsui (Aomori Prefectural Institute of Public Health and Environment), Masaki Takahashi (Research Institute for Environmental Sciences and Public Health of Iwate Prefecture), Hiroshi Uemura (Miyagi Prefectural Institute of Public Health and Environment), Masao Sekine (Sendai City Institute of Public Health), Hiroyuki Saito (Akita Research Center for Public Health and Environment), Tatsuya Ikeda (Yamagata Prefectural Institute of Public Health), Keiko Tsukada (Fukushima Prefectural Institute of Public Health), Miyako Kon (Niigata Prefectural Institute of Public Health and Environmental Sciences), Kazunari Yamamoto (Niigata City Institute of Public Health and Environment), Setsuko Fukaya (Ibaraki Prefectural Institute of Public Health), Teruko Oogane (Tochigi Prefectural Institute of Public Health and Environmental Sciences), Miho Ikegaya (Utsunomiya City Institute of Public Health and Environment Science), Hiroyuki Tsukagoshi (Gunma Prefectural Institute of Public Health and Environmental Sciences), Shinichi Shimada (Saitama Institute of Public Health), Yuka Uno (Saitama City Institute of Health Science and Research), Hiromi Maru (Chiba Prefectural Institute of Public Health), Hajime Yokoi (Chiba City Institute of Health and Environment), Mami Nagashima (Tokyo Metropolitan Institute of Public Health), Sumi Watanabe (Kanagawa Prefectural Institute of Public Health), Chiharu Kawakami (Yokohama City Institute of Health), Hideaki Shimizu (Kawasaki City Institute of Public Health), Megumi Takeuchi (Yokosuka Institute of Public Health), Noriko Sagiya (Sagamihara City laboratory of Public health), Masayuki Oonuma (Yamanashi Institute for Public Health), Yuka Azegami (Nagano Environmental Conservation Research Institute), Yuichiro Okamura (Nagano City Health Center), Toshihiro Yamada (Shizuoka Institute of Environment and Hygiene), Nona Shibahara (Shizuoka City Institute of Environmental Sciences and Public Health), Yukie Suzuki (Hamamatsu City Health Environment Research Center), Masatsugu Obuchi (Toyama Institute of Health), Hiroe Kodama (Ishikawa Prefectural Institute of Public Health and Environmental science), Eiko Hirano (Fukui Prefectural Institute of Public Health), Tsuyoshi Kuzuguchi (Gifu Prefectural Institute of Health and Environmental Sciences), Yuko Matsubara (Gifu Municipal Institute of Public Health), Yoshihiro Yasui (Aichi Prefectural Institute of Public Health), Shinichiro Shibata (Nagoya City Public Health Research Institute), Takuya Yano (Mie Prefecture Health and Environment Research Institute), Hiromi Kodama (Shiga Prefectural Institute of Public Health), Tohru Ishizaki (Kyoto Prefectural Institute of Public Health and Environment), Mayumi Konno (Kyoto City Institute of Health and Environmental Sciences), Satoshi Hiroi (Osaka Prefectural Institute of Public Health), Hideyuki Kubo (Osaka City Institute of Public Health and Environmental Sciences), Kiyoko Uchino (Sakai City Institute of Public Health), Tomohiro Oshibe (Hyogo Prefectural Institute of Public Health and Consumer Sciences), Tomoko Suga (Kobe Institute of Health), Shinya Kawanishi (Himeji City Institute of Environment and Health), Yasuhiro Hagihara (Amagasaki City Institute of Public Health), Yoshiteru Kitahori (Nara Prefectural Institute for Hygiene and Environment), Fumio Terasoma (Wakayama Prefectural Research Center of Environment and Public Health), Hidenobu Ekawa (Wakayama City Institute of Public Health), Yoshiaki Kimura (Tottori Prefectural Institute of Public Health and Environmental Science), Mieko Wada (Shimane Prefectural Institute of Public Health and Environmental Science), Mitsutaka Kuzuya (Okayama Prefectural Institute for Environmental Science and Public Health), Shinichi Takao (Center for Public Health and Environment, Hiroshima Prefectural Technology Research Institute), Miwako Yamamoto (Hiroshima City Institute of Public Health), Shoichi Toda (Yamaguchi Prefectural Institute of Public Health and Environment), Yasuhiro Nishino (Tokushima Prefectural Centre for Public Health and Environmental Sciences), Hiroya Komoda (Kagawa Prefectural Research Institute for Environmental Sciences and Public Health), Satomi Aoki (Ehime Prefecture Institute of Public Health and Environmental Science), Tae Taniwaki (Kochi Public Health and Sanitation Institute), Hideaki Yoshitomi (Fukuoka Institute of Health and Environmental Sciences), Keiko Kajiyama (Fukuoka City Institute for Hygiene and the Environment), Naomi Kawabe (Kitakyushu City Institute of Environmental